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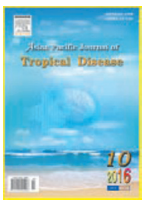

Parasitological research doi: 10.1016/S2222-1808(16)61131-3

High infection of *Anaplasma* and *Ehrlichia* spp. among tick species collected from different geographical locations of Iran

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ARTICLE INFO

Article history:
Received 28 Jul 2016
Received in revised form 12 Aug, 2nd revised form 15 Aug 2016
Accepted 21 Aug 2016
Available online 25 Aug 2016

Keywords:
Tick
Anaplasma ovis
Ehrlichia spp.
PCR
Iran

ABSTRACT

Objective: To ascertain the prevalence of the *Anaplasma/Ehrlichia* infections in tick population within four provinces of Iran.

Methods: A total of 384 tick specimens were collected from domestic animals inhabiting in four provinces (East Azerbaijan, Gilan, South Khorasan and Yazd). Specimens were identified based on morphological analysis. The detection of *Anaplasma* spp./*Ehrlichia* spp. within tick samples was carried out by nested PCR amplification of the 16S ribosomal RNA gene accompanied by DNA sequencing and analysis for verification.

Results: A total of 10 tick species were identified as follows: *Ornithodoros lahorensis* (*O. lahorensis*) (44.8%), *Hyalomma dromedarii* (15.6%), *Dermacentor marginatus* (13.5%), *Hyalomma anatolicum* (11.2%), *Hyalomma asiaticum* (5.7%), *Hyalomma marginatum* (4.9%), *Rhipicephalus sanguineus* (2.3%), *Hyalomma detritum* (1.0%), *Dermacentor niveus* (0.5%) and *Argas persicus* (0.3%). The percentage distribution of *Anaplasma/Ehrlichia* was 55.5% (213) across 384 studied ticks.

Conclusions: To the best of our knowledge, this is the first report of *Anaplasma ovis* infection in *O. lahorensis* in Iran. We also conjecture the prevalence of *Ehrlichia* spp. in Yazd Province based on sequencing results; also, it is suggested that *O. lahorensis* is a potential vector in the studied area. This survey highlights the importance of Argasidae family to verify and correlate their threat in causing anaplasmosis and other diseases in animals.

1. Introduction

Anaplasmosis and ehrlichiosis, caused by *Anaplasma* and *Ehrlichia* species respectively, are major tick borne infectious diseases in domestic animals and to a lesser extent in humans[1]. The infections contribute to significant losses in animal productivity and are deemed potentially fatal[2].

Anaplasma and *Ehrlichia* genera (order: Rickettsiales, family: Anaplasmataceae) comprise of Gram-negative bacteria species which are obligatory intracellular pathogens. They display similar morphologies, genome organizations and life cycles marked by replication inside membrane-bound vacuoles within host cells forming morulae. Their targets in vertebrate hosts range from erythrocytes, granulocytes, monocytes and platelets to blood vessel endothelial cells whereas in invertebrate hosts, the ticks reside in salivary glands and midgut[3].

Ticks (class: Arachnid) are blood feeding ectoparasites which are involved in transmission of several infections and diseases[4,5]. A total of 896 tick species across three families have been recognized in the world[6]. In most regions of Iran, the dominant tick genera responsible for infestation in ruminants belong to *Hyalomma, Rhipicephalus, Haemaphysalis, Ixodes*[7-9]. The species pertaining to the above mentioned genera have already been associated with vectors of the severe forms of protozoan diseases (*Babesia* and

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The journal implements double-blind peer review practiced by specially invited international editorial board members.

Theileria) and viral diseases (Crimean-Congo hemorrhagic fever) in Iran[10-13]. Past surveys implied that *Anaplasma marginale* (*A. marginale*) (cattle), *Anaplasma ovis* (*A. ovis*) (sheep, goat) and *Ehrlichia canis* (dogs) are widespread in several parts of Iran (Table 1). It also appears that *Rhipicephalus sanguineus* (*R. sanguineus*) is the main vector of *A. ovis* along with *Hyalomma* spp., *Dermacentor* spp. and *Ixodes* spp. (Table 1).

Currently, molecular detection of *Anaplasma/Ehrlichia* in host and vector is achieved by reverse line blot hybridization-PCR, species specific nested PCR, or full length PCR of *GroEL*, *msp4*, 16S rRNA and 18S rRNA genes often followed by gene sequencing/restricted fragment length polymorphisms (RFLP)[14,15]. These methods are far more sensitive and specific than the earlier diagnostic methods of microscopic examination of Giemsa-stained blood smears and surface protein specific antibody based ELISA[16].

Anaplasmosis and ehrlichiosis are impediments to domestic breeding of ruminants in Iran. Therefore, we decided to survey the *Anaplasma/Ehrlichia* infection in ticks from parts of Iran which have distinct geographic and climate profiles and so far have never been surveyed before. We conducted our field study in four provinces and recovered 384 tick samples from domestic animals. We determined the presence of *Anaplasma/Ehrlichia* species in the tick samples by nested PCR of 16S rRNA gene fragment followed by sequencing of a subset of the positive cases.

2. Materials and methods

2.1. Tick collection and identification

The study was carried out in four different locations with different climate regimes, namely, Yazd (hot and dry climate), Gilan (humid subtropical climate in forest and beach area), South Khorasan (harsh hot and dry climate in sand dunes region) and East Azerbaijan Provinces (cool and dry climate in mountainous region) in Iran (Figure 1). The sampling period commenced in January 2011 and ended in December 2011. Sampling was undertaken in different types of livestock found in stables situated in randomly chosen villages.

Figure 1. The areas of study including East Azerbaijan, Gilan, Yazd and South Khorasan Provinces marked on the map of Iran as orange, green, cream and brown respectively.

After collection, ticks were transported to the laboratory of the Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Science. The tick species were identified morphologically by microscopy using the available tick identification taxonomy keys[17-20].

Table 1
List of *Anaplasma/Ehrlichia* spp. surveyed in ticks and vertebrates across different regions in Iran.

Places	Tick genera (*Anaplasma* or *Ehrlichia* species)	Vertebrate (*Anaplasma* or *Ehrlichia* species)	Method of detection	Reference
Ghaemshahr Mazandaran Province	*Ixodes ricinus* (*Anaplasma phagocytophilum*)	-	PCR-RFLP of 16S rRNA gene	[21]
Seven regions	Ticks (*Ehrlichia ovina*)	Sheep (*A. ovis*)	PCR-sequencing 16S and 18S rRNA gene	[22]
Mashhad Suburb, Khorasan Province	-	Cattle (*A. marginale*), sheep (*A. ovis*) and goat (*A. ovis*)	Microscopy Giemsa-staining	[23]
Golestan and Khorasan Razavi Provinces	-	Goats (*A. ovis* and *A. marginale*)	PCR-RFLP of *msp4* gene	[14]
Isfahan	-	Cattle (*Anaplasma phagocytophilum*, *A. marginale* and *A. bovis*)	Nested PCR of 16S rRNA gene	[15,24]
Ahvaz, Khuzestan Province	-	Sheep (*A. ovis* and *A. marginale*)	PCR-RFLP of *msp4* gene	[25]
South West region of Iran (Fars Province)	-	Cattle (*A. marginale*)	Microscopy Gram staining	[26]
Ardebil Province (North-West Iran)	*Rhipicephalus* (*A. ovis*, *Ehrlichia* spp.), *Hyalomma* (*A. ovis*, *Ehrlichia* spp.), *Dermacentor* (*A. ovis*, *Ehrlichia* spp.), *O. lahorensis* (N.F.)	Dogs (N.S.)	Nested PCR-sequencing 16S rRNA gene	[27]
Ghaemshahr Mazandaran Province	*R. sanguineus* (*A. ovis*), *Ixodes ricinus* (*A. ovis*), *Rhipicephalus annulatus* (*A. bovis*)	Human (*A. ovis*), sheep (*A. ovis*, *A. bovis* and *Anaplasma centrale*)	Nested PCR-sequencing 16S rRNA gene	[1]
Qom Province	*R. sanguineus* (*A. ovis*), *Hyalomma* (N.F.)	Sheep and camel (N.S.)	Nested PCR 16S rRNA gene	[28]
Alborz and Tehran	-	Dogs (Ehrlichia canis)	Nested PCR-sequencing 16S rRNA gene	[29]

A. bovis: *Anaplasma bovis*; *O. lahorensis*: *Ornithodoros lahorensis*; N.F.: Not found; N.S.: Not surveyed.

2.2. Molecular method for detection of Anaplasma/Ehrlichia infections in ticks

2.2.1. DNA isolation, Nested PCR amplification and sequencing

Each tick was immersed in 70% ethanol for 15 min; air dried and kept in separate 1.5 mL Eppendorf tubes to avoid cross contamination. Each specimen was incubated in liquid nitrogen for 5 min followed by grinding using mortar and pestle. DNA was extracted using a rapid genomic DNA isolation kit (Molecular Biological System Transfer, Iran) as per the manufacturer's instructions. A 524 bp fragment of the 16S ribosomal RNA gene was amplified by nested-PCR. The primer pair used for first PCR step was Ehr1 5´-GAACGAACGCTGGCGGCAAGC-3´ and Ehr2 5´-AGTA[T/C]CG[A/G]ACCAGATAGCCGC-3´. Nested PCR was performed on the PCR product generated in first step using second primer pair set, Ehr3 5´-TGCATAGGAATCTACCTAGTAG-3´ and Ehr4 5´-CTAGGAATTCCGCTATCCTCT-3´ which yielded a 524 bp product[30]. The PCR procedure followed in this study is mostly same as described in Rar *et al*. except with slight modifications in the annealing temperature values[30]. The PCR amplification was performed with 25 μL reaction mixtures containing 2.5 μL PCR buffer, Tris-HCl (10×) (pH 9.0), 0.75 μL $MgCl_2$, 0.5 μL dNTPs, 0.2 μL Taq DNA polymerase, 1 μL of each forward and reverse primers (final concentration: 0.5 μmol/L), 1 μL of reverse primer (final concentration: 0.5 μmol/L), 2 μL of DNA extract and rest distilled water in an automatic DNA thermo-cycler (Peqlab, Germany). The PCR conditions were set as follows: initial genomic DNA denaturation at 94 ˚C for 5 min followed by 35 cycle reaction program (denaturing at 94 ˚C for 1 min, annealing at 60 ˚C for 1 min and elongation at 72 ˚C for 1 min) and final extension at 72 ˚C for 7 min. For few reactions, the annealing temperature was optimized at 57 ˚C. Genomic DNA of *Anaplasma* species obtained from Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran was used as positive control while double distilled water served as negative control[24]. PCR products were visualized in 1.2% agarose gels stained with ethidium bromide under UV light (Bioimagine System, Syngene, UK). Amplified products were purified by using the QIAquick gel extraction kit (Qiagen, Germany).

Thirty samples out of 213 PCR positive samples were selected for DNA sequencing. The selection was made in such a way so that at least, one infected specimen of each individual tick species (in proportion to infected sample size) from each province would be represented in the final sequencing set. Sequencing of final nested PCR products was performed by Genfanavaran, Iran. Sequences were manually checked to correct any sources of error or ambiguities if present. Homologies with the available sequence data in GenBank were checked using BLAST analysis software. Finally to confirm the BLAST results, the sequenced 16s rRNA gene sequences were aligned by performing multiple pair alignments with corresponding gene orthologs from all *Anaplasma* spp. using the ClustalW software.

3. Results

A total of 384 ticks were collected and after subsequent morphological analysis, 10 species were identified across two families and five genera as follows (Table 2) comprising *Ornithodoros lahorensis* (*O. lahorensis*) (44.8%), *Hyalomma dromedarii* (*H. dromedarii*) (15.6%), *Dermacentor marginatus* (*D. marginatus*) (13.5%), *Hyalomma anatolicum* (*H. anatolicum*) (11.2%), *Hyalomma asiaticum* (*H. asiaticum*) (5.7%), *Hyalomma marginatum* (*H. marginatum*) (4.9%), *R. sanguineus* (2.3%), *Hyalomma detritum* (1.0%), *Dermacentor niveus* (*D. niveus*) (0.5%) and *Argas persicus* (*A. persicus*) (0.3%) (Figire 2).

The nested PCR of 524 bp fragment 16S rRNA gene fragment in 384 genomic DNA samples confirmed the presence of *Anaplasma/Ehrlichia* species in 213 (55.5%) cases (Table 2). The percentage distribution of *Anaplasma/Ehrlichia* infected specimens across East Azerbaijan, Gilan, South Khorasan and Yazd Provinces were 71%, 47%, 44% and 41% respectively (Figire 2). Overall statistics for each species defined in terms of infected counts *vs*. total sample size accompanied by percentage abundance of infected cases was as follows: *A. persicus* (1/1, 100%), *D. niveus* (1/2, 50%), *D. marginatus* (24/52, 46.1%), *H. anatolicum* (19/43, 44.2%), *H. asiaticum* (8/22, 36.4%), *H. detritum* (2/4, 50%), *H. dromedarii* (26/60, 43.3%), *H. marginatum* (7/19, 36.8%), *O. lahorensis* (120/172, 69.8%) and *R. sanguineus* (5/9, 55.6%) (Figire 2).

The origin of sequenced data was determined by NCBI-BLAST

Table 2

Distribution of tick specimens and the frequencies of infected samples according to their gender in the four provinces.

No.	Tick species [*n* (%)]	Province	Number of samples collected	Gender distribution of samples collected (male/female)	Number of infected samples as confirmed by nested- PCR (%)	Gender distribution of infected samples (male/female)	Number of infected samples sequenced (species identified)
1.	*A. persicus* [1 (100.0)]	South Khorasan	1	1/0	1 (100.0)	1/0	1 (*A. ovis*)
2.	*D. marginatus* [52 (46.1)]	Gilan	52	31/21	24 (46.1)	14/10	2 (*A. ovis*)
3.	*D. niveus* [2 (50.0)]	South Khorasan	2	2/0	1 (50.0)	1/0	1 (*A. ovis*)
4.	*H. anatolicum* [43 (44.2)]	East Azerbaijan	3	3/0	3 (100.0)	3/0	1 (*A. ovis*)
		South Khorasan	22	10/12	9 (40.9)	4/5	1 (*A. ovis*)
		Yazd	18	10/8	7 (38.9)	4/3	1 (*Ehrlichia* spp.)
5.	*H. asiaticum* [22 (36.4)]	South Khorasan	5	2/3	3 (60.0)	0/3	1 (*A. ovis*)
		Yazd	17	1/16	5 (29.4)	1/4	1 (*Ehrlichia* spp.)
6.	*H. detritum* [4 (50)]	South Khorasan	2	1/1	1 (50.0)	1/0	1 (*A. ovis*)
		Yazd	2	2/0	1 (50.0)	1/0	1 (*Ehrlichia* spp.)
7.	*H. dromedarii* [60 (43.3)]	South Khorasan	8	6/2	4 (50.0)	2/2	1 (*A. ovis*)
		Yazd	52	20/32	22 (42.3)	10/12	2 (*Ehrlichia* spp.)
8.	*H. marginatum* [19 (36.8)]	South Khorasan	12	4/8	4 (33.3)	2/2	1 (*A. ovis*)
		Yazd	7	6/1	3 (42.8)	3/0	1 (*Ehrlichia* spp.)
9.	*O. lahorensis* [172 (69.8)]	East Azerbaijan	169	78/98	119 (70.4)	56/63	10 (A. ovis)
		South Khorasan	3	3/0	1 (33.3)	1/0	1 (*A. ovis*)
10.	*R. sanguineus* [9 (55.6)]	South Khorasan	4	4/0	1 (25.0)	1/0	1 (*A. ovis*)
		Gilan	1	1/0	1 (100.0)	1/0	1 (*A. ovis*)
		Yazd	4	3/1	3 (75.0)	3/0	1 (*Ehrlichia* spp.)
	Total [384 (55.5)]		384	181/203	213 (55.5)	109/104	30

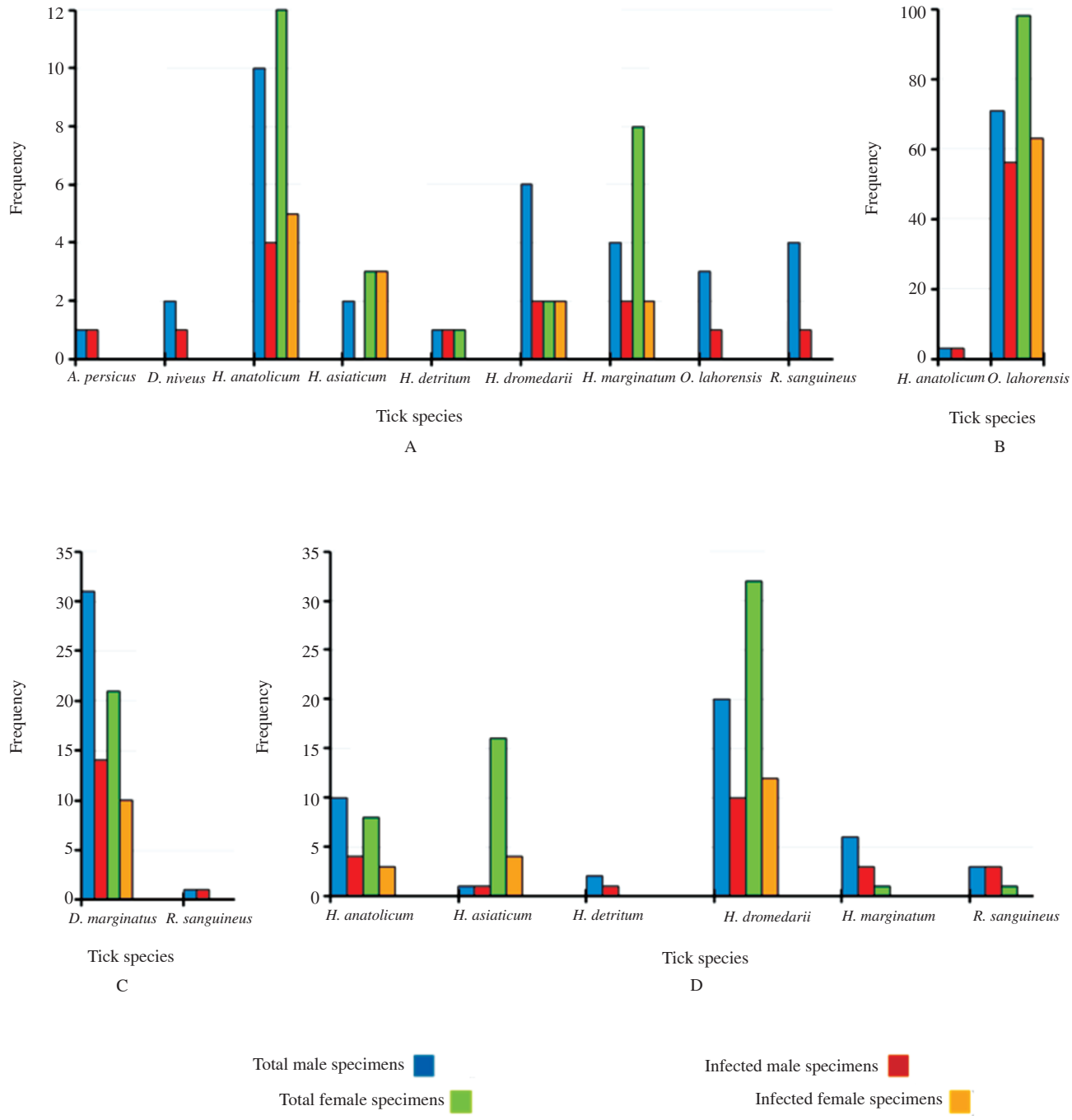

Figure 2. Distribution of tick species and *Anaplasma/Ehrlichia* infection prevalence in each of the four provinces (total tick count; infected tick percentage.

A: South Khorasan (59; 42.4%); B: East Azerbaijan (172; 70.9%); C: Gilan (53; 47.2%); D: Yazd (100; 41%).

software which identified 20 sequences belonging to *A. ovis* while the remaining 10 mapped to *Ehrlichia* spp. (Table 2). The *A. ovis* positive sequences were 100% identical with those reported from Russia, Tibet, Iran, China, Netherland and Sweden having corresponding GenBank Accession numbers as KC484563, JQ917886, JF514503-JF514511, EF587237, AF318945 and AY837736 respectively. While the positive *Ehrlichia* spp. sequences were 100% identical to those reported from China, Tibet, Africa, Vietnam and Iran having corresponding GenBank Accession numbers as DQ324547.1, AF414399.1, AF311968.1, AF497581.1 and JN626225.1 respectively.

4. Discussion

The Ixodidae family (hard ticks) are major vectors of *Anaplasma* and *Ehrlichia* worldwide[31,32]. Among Ixodidae family (hard ticks), *Rhipicephalus* spp. and *Hyalomma* spp. are ubiquitous in Iran[9,33]. In our study, *Hyalomma* species, dominant in South Khorasan and Yazd, accounted for 148 specimens with 41.9% infection rate[34]. However, *R. sanguineus* showcased a meager population of 2.3% even though it was prevalent and infected in three provinces (Table 1). South Khorasan and Yazd being neighbors share similar geographical attributes; therefore they displayed a similar distribution of tick species even though their individual abundance varied. Unexpectedly, a significant disparity was discerned when the sequencing results of infected tick species revealed the presence of *Ehrlichia* spp. solely in Yazd as opposed to the manifestation of *A. ovis* in South Khorasan, Gilan and East Azerbaijan. *D. marginatus* was the prominent species in Gilan with an infection rate of 46.2%

(Table 2).

O. lahorensis and *A. persicus* belong to Argasidae (soft tick) family, which flourish in colder climates. A survey undertaken in West Azerbaijan revealed that *O. lahorensis* and *A. persicus* are the predominant soft ticks in the region[35]. However in East Azerbaijan, *O. lahorensis* being the neighbor of West Azerbaijan was found to be the most abundant species (169 specimens) in our study with no instance of *A. persicus* (Table 2). Since we did not gather seasonal information, we cannot not rule out the possibility that the high abundance of *O. lahorensis* reported in East Azerbaijan might be due to the fact that the sampling was done in winter since former was predominant in West Azerbaijan only during winters[35]. South Khorasan on the other hand exhibited a scanty habitation of *O. lahorensis* and *A. persicus* (Figure 2).

The salient aspect which was deduced from this work was apart from the most abundant species, *O. lahorensis* demonstrated the maximum infection rate of 70.9%. Moreover, *A. ovis* was responsible for the infections in all of the 11 sequenced *O. lahorensis* samples (Table 2). In an earlier study, *O. lahorensis* specimens captured from dogs in Ardabil Province of Iran were negative for *Anaplasma/ Ehrlichia* spp.[27]. Consequently, this is the first ever report which provides molecular evidence to confirm *O. lahorensis*, a member of Argasidae (soft tick) family, to be the natural vector of *A. ovis* in Iran. It also places *O. lahorensis* at par along with other major *A. ovis* vectors, namely, *R. sanguineus*, *Hyalomma* spp. and *D. marginatus*[1,27,36]. *O. lahorensis* is known to cause tick paralysis in sheep and is also a vector of *Borrelia persica*, *Brucella* and Crimean-Congo hemorrhagic fever virus in Iran while in Central Asia, it carries *Theileria ovis*[37-41]. It will be interesting to discover the prevalence of other pathogens in *O. lahorensis* infested regions of Iran.

A. ovis mainly parasitizes erythrocytes of ovine ruminants, *i.e.* sheep and goats, and surprisingly its presence has been detected in humans as well in Iran[1,14,16,22,23,25,42]. The infected animals experience an acute phase of marked decline in body weight, fever, anaemia, reduction in milk production and can be lethal if the disease is unchecked. The symptoms of anaplasmosis depend on age, the general condition of the animals and their breed[43]. A recent study carried on prevalence of *A. ovis* in sheep and goat from Mashhad region of Iran reported that former shows seasonal variation, with infection peaking during summers and subsiding during winters. Moreover, the infected animals were asymptomatic for anaplasmosis disease[23]. Nevertheless, the infected animals are susceptible to disease development and usually act as lifelong reservoirs. Argasidae are resistant to starvation and can survive for several years without feeding[44]. Furthermore, their diapause periods grant them great flexibility in their developmental cycles[45]. Thus, even though a high infestation of *A. ovis* in *O. lahorensis* may not necessarily lead to disease, it is a matter of great concern owing to the perpetual maintenance of *A. ovis* reservoir within the tick and their animal hosts.

To conclude, our finding suggests that *O. lahorensis* (soft tick) could be the major vector of *A. ovis* especially in regions bearing cold climates where soft ticks thrive better than hard ticks. In addition, despite being able to sequence only few tick specimens, we infer that *Ehrlichia* spp. is prevalent in Yazd Province while *A. ovis* infection is conspicuous in the other three provinces. This study justifies the need of further investigation into the incidences of anaplasmosis and ehrlichiosis in vertebrate hosts in Iran.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We would like to thank the staff of Health Research Institute for their precious contributions. We are thankful to Miss Samira Tajeddini for her unconditional help. This work was supported by the School of Public Health of Tehran University of Medical Sciences.

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